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Acid aspiration provokes the pneumonia caused by multidrug-resistant *Acinetobacter baumannii* in BALB/c mice

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SUMMARY

Objective: To determine whether acid aspiration provokes the development of multidrug-resistant *Acinetobacter baumannii* (MDRAB) pneumonia in its host.
Methods: Groups of mice were inoculated intratracheally (IT) with 50 μl of 0.1 N HCl and 1×10^8 colony-forming units (CFU) Ab396 (A + Ab group), or 50 μl of 0.1 N HCl and 20 μl of 0.9% saline (A + S group), or 20 μl of 0.9% saline and 1×10^8 CFU of Ab396 (S + Ab group), or 50 μl of 0.9% saline and 20 μl of 0.9% saline (S + S group). Cytokines, bacterial loads in the bronchoalveolar lavage fluid (BALF), lung permeability, histopathology of the lungs, and survival rates were evaluated.
Results: Only the A + Ab mice developed extensive Ab396 pneumonia and had significantly elevated bacterial loads, increased lung leakage, and lower levels of tumor necrosis factor alpha (TNF-α) compared with the other three groups ($p < 0.05$, Mann–Whitney *U*-test). Moreover, a strong synergistic effect ($p < 0.05$, two-way analysis of variance) was observed between the acid induction and Ab396 infection, resulting in lung injury and an unfavorable survival outcome.
Conclusions: Lung injury caused by acid aspiration provoked secondary MDRAB pneumonia; also synergistic effects between acid aspiration and Ab396 infection resulted in a detrimental outcome in the infected mice.

1. Introduction

Acinetobacter baumannii is a non-fermenting Gram-negative bacterium that is widely distributed in the natural environment.[1] It is also an important opportunistic pathogen in a variety of nosocomial infections resistant to most current antibiotic therapies.[2,3] Nosocomial pneumonia caused by multidrug-resistant *A. baumannii* (MDRAB) has been increasingly reported worldwide,[4,5] resulting in high mortality rates (30–75%) in patients, particularly those in hospital intensive care units.[6,7] Several studies have identified a number of risk factors for the occurrence of MDRAB pneumonia, including tracheostomy, lasting ventilation, dialysis, *Stenotrophomonas maltophilia* colonization, parenteral nutrition, enteric feeding, and prior antimicrobial therapy.[8–10] However, the methodological heterogeneity of these studies makes it difficult to derive clear conclusions about the pathogenesis of MDRAB pneumonia.

Gastric aspiration leads to a chemical burn with an intense inflammatory reaction of the pulmonary parenchyma.[11] It is known to occur easily in unconscious, ventilated hospitalized patients or in patients who have received general anesthesia.[11–14] Moreover, a 6- to 21-fold increase in the incidence of pneumonia after gastric aspiration in intubated patients has been reported.[15] In fact, gastric aspiration can facilitate secondary bacterial infection of the lungs and also lead to the development of acute respiratory distress syndrome (ARDS).[16]

A. baumannii is considered an opportunistic pathogen. Nevertheless, a series of insults to a host may augment the host's immune response and lead to significant lung injury.[17,18] The initial acid aspiration triggers a marked inflammatory response,

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which can negatively impact a host's outcome following a subsequent infectious challenge. Thus, we hypothesized that lung injury after acid aspiration would provoke the development of secondary MDRAB pneumonia in the host. To test this hypothesis, we conducted a two-hit experimental design that combined the well-established acid aspiration-induced model and a novel secondary MDRAB infection protocol to determine whether prior pulmonary acid aspiration is an important risk factor that complicates MDRAB pneumonia.

2. Materials and methods

2.1. Microorganism

Ab396, an MDRAB strain that is only susceptible to colistin methanesulfonate, was isolated from the bloodstream of a patient. Aliquots were prepared and frozen at −80 °C until use. Details of the minimum antibiotic inhibitory concentration of this strain are presented in our previous report.[19]

2.2. Mice

Six- to eight-week-old inbred female BALB/c mice weighing 20–22 g were purchased from the Animal Center of the National Science Council, Taipei, Taiwan, for this study. Prior to the experiments, the mice were allowed to adjust to the Animal Center of Chi-Mei Medical Center for 5–7 days, with food and water supplied ad libitum.[19]

2.3. Determination of the sub-lethal dose for the intratracheal (IT) inoculum of Ab396

The median lethal dose (LD_{50}) for Ab396 in mice was determined to be 2.5×10^8 CFU using previous methods.[20] To determine the sub-lethal dose for this study, mice ($n = 6$ per group) were inoculated IT with Ab396 concentrations of 1×10^9, 1×10^8, and 1×10^7 CFU in 20 μl 0.9% saline. Fatality rates on the third day were 100%, 0%, and 0%, respectively. Thus, a sub-lethal dose of 1.0×10^8 CFU of Ab396 per mouse was chosen for all subsequent experiments.

2.4. Establishment of lung injury after 0.1 N HCl instillation (the first hit)

The acute lung injury model induced by 0.1 N HCl was performed as described previously.[21] To ascertain that acute lung injury was established, 12 mice were treated with IT 50 μl (2.5 ml/kg) of 0.1 N HCl and six mice with 0.9% saline. The trachea was cannulated with a 25-gauge needle and 1 ml 0.9% saline was infused into the lungs; between 0.9 and 0.95 ml of BALF was retrieved and the cells were immediately pelleted by centrifugation at 500 *g* for 5 min at 4 °C. Leukocyte concentrations, obtained at 6 h after 0.1 N acid instillation, were determined by resuspending the cell pellets in 1 ml of phosphate-buffered saline (PBS, pH 7.2), using the Multisizer 3 Coulter Counter (Beckman Coulter). A cytoslide was prepared with 5×10^4 leukocytes using a Cytospin 3 cytocentrifuge (Shandon, Pittsburgh, PA, USA) and stained with Diff-Quik reagents (Baxter, Miami, FL, USA). The total and differentiated cellular counts in BALF were assayed. Additionally, six healthy mice without any treatment were enrolled as the control group. The histopathology of the lungs was examined at 24 h in another four mice treated with 0.1 N HCl IT.

2.5. Experimental procedure for the two-hit Ab396 pneumonia model

A two-hit experiment was adapted from previous methods.[19,21] Briefly, mice were anesthetized with 70 mg/kg sodium pentobarbital by intraperitoneal injection and supported on a frame. The airway was externally illuminated by a lamp, and vocal folds were exposed. A micropipette was passed down the pharynx, and its tip was inserted between the vocal folds to administer the first hit of 50 μl 0.1 N HCl IT. After 5 min, the second hit of 1.0×10^8 CFU of Ab396 in 20 μl 0.9% saline (A + Ab group) or 20 μl 0.9% saline only (A + S group) was delivered. A third group of mice was treated with an initial IT challenge of 50 μl 0.9% saline, followed by IT administration of 1.0×10^8 CFU of Ab396 in 20 μl 0.9% saline (S + Ab group) after the same 5-min time interval. Healthy mice given an initial IT dose of 50 μl 0.9% saline, followed by IT administration of 20 μl 0.9% saline (S + S group), were enrolled as a control group.

2.6. Survival study

In the S + S, A + S, A + Ab, and S + Ab groups ($n = 8$ for each group), the survival rates were recorded every day for 6 days after the induction of secondary Ab396 pneumonia.

2.7. Bacterial loads and cytokine levels in BALF

Fifty treated mice in the S + S, A + S, S + Ab, and A + Ab groups were tested. Briefly, mice were anesthetized and the trachea cannulated with a 25-gauge needle, as in our previous report.[22] One milliliter of 0.9% saline was infused into the lungs. Between 0.9 and 0.95 ml BALF was retrieved from each mouse, and the cells were immediately pelleted by centrifugation at 3500 *g* for 10 min at 4 °C. Bacterial loads were measured in the BALF at 3, 6, 12, 24, and 48 h after IT inoculation of Ab396, as previously reported.[19] The cell-free supernatant was stored at −80 °C for analysis of cytokine content. Levels of tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, macrophage inflammatory protein 2 (MIP-2), and IL-10 in the BALF were assessed at the same time points as in the previous report.[22]

2.8. Lung injury and interaction between the acidic environment and the Ab396 infection in the lungs

Lung permeability was assayed to measure the lung injury in the pneumonia model. The Evans blue dye (EBD) titer in the BALF was assayed to determine lung permeability, as in our previous report.[23] Briefly, the trachea of each mouse was cannulated with a 25-gauge needle. Evans blue dye (50 mg/kg) was administered via the tail vein of the mouse, and 1 ml of 0.9% saline was infused into the lungs via the tracheal needle. BALF was collected from the S + S, A + S, S + Ab, and A + Ab mice at 6, 24, and 48 h (eight surviving mice were collected and tested at each time point) for assay. The extravasated EBD concentration in the BALF was calculated against a standard curve to determine the change in lung permeability. Also, the interaction effect between the acidic environment and the presence of Ab396 on the permeability of the lungs from 6 to 48 h was assayed.

2.9. Histopathological study of the lungs

The lungs were harvested at 6, 12, 24, and 48 h after the second hit from the mice in the A + Ab, A + S, and S + Ab groups, as well as from the healthy mice ($n = 4$ for each time point). Tissues were fixed in 10% formaldehyde, embedded in paraffin, sectioned, stained with hematoxylin–eosin (HE), and examined by light microscopy. The micrographs of the HE-stained lung tissue were reviewed by a pathologist.

2.10. Statistical analysis

The planned comparisons for the measured parameters (i.e., cell counts in BALF, lung permeability, bacterial counts, and cytokine

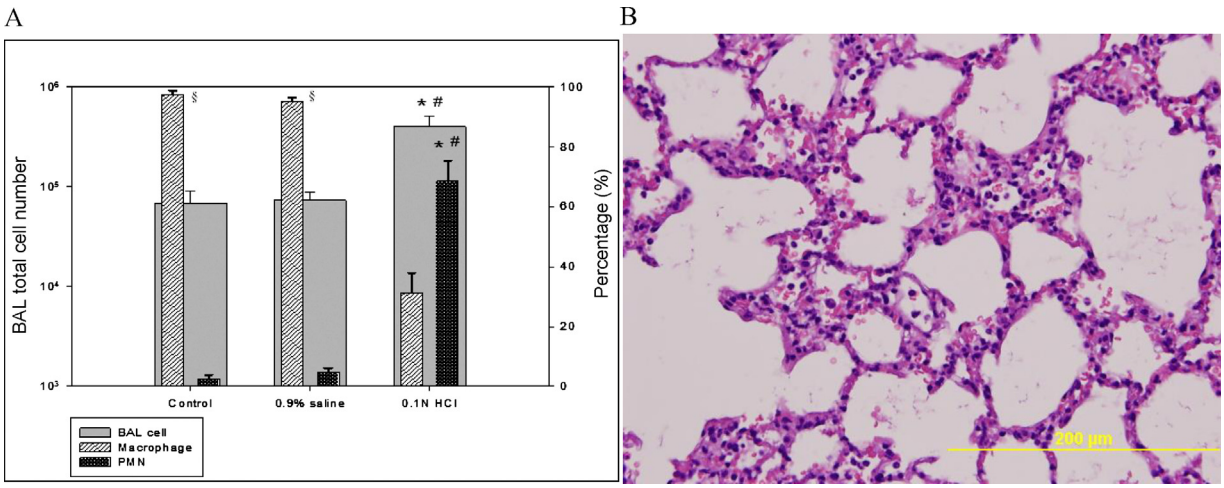

Figure 1. Establishment of lung injury for the first hit after 0.1 N HCl aspiration. (A) Total BALF cell counts, percent macrophages, and PMN levels in mice 6 h after intratracheal injection of 0.1 N HCl or 0.9% saline, and in healthy controls. (B) Representative lung histology slide (hematoxylin and eosin stain) of the lungs from the mice at 24 h after 0.1 N HCl instillation.
*Significantly higher total cell numbers or percentage of PMN in the 0.1 N HCl group compared with the healthy control group.
#Significantly higher total cell numbers or percentage of PMN in the 0.1 N HCl group compared with 0.9% saline group.
§Significantly higher percentage of macrophages in the healthy control and 0.9% saline groups compared with the 0.1 N HCl group.

levels) were performed between groups by Mann–Whitney *U*-test. The interaction effect between acid and Ab396 on the lung injury were explored by stratified analysis using the Mann–Whitney *U*-test among the groups with Ab396 and without Ab396, respectively. A full model of two-way analysis of variance (ANOVA) was additionally performed to examine the statistical significance of the cross-product term of acid and Ab396. The mortality rates over time were compared between the four groups using the log-rank test. A *p*-value of less than 0.05 was considered statistically significant. Means are shown to describe the central tendency; standard deviations to describe the data dispersion. All data were analyzed with SPSS software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cellular analysis in BALF and histopathology of the lungs after IT 0.1 N HCl instillation

The total BALF cell counts in mice treated with 0.9% saline were similar to those of the healthy control mice. However, 0.1 N HCl-treated mice had a significantly higher level of total BALF cell numbers ($4.0 \pm 1.12 \times 10^6$) than 0.9% saline-treated mice ($7.3 \pm 1.62 \times 10^5$) and healthy control mice ($6.9 \pm 2.23 \times 10^5$) at 6 h after acid instillation (vs. 0.9% saline and healthy control mice, all $p < 0.05$ by Mann–Whitney *U*-test). Moreover, a significantly higher percentage of polymorphonuclear leukocytes (PMN) ($68.7 \pm 6.7\%$) and lower percentage of macrophages ($31.3 \pm 6.7\%$) were found in 0.1 N HCl-treated mice compared to the saline-treated mice (PMN $4.8 \pm 1.4\%$, macrophages $95.2 \pm 1.4\%$) and healthy control mice (PMN $2.5 \pm 1.3\%$, macrophages $97.5 \pm 1.3\%$) (vs. 0.9% saline, healthy mice group, all $p < 0.05$ by Mann–Whitney *U*-test) (Figure 1A). Pathohistologic findings in the lungs of mice at 24 h after 0.1 N HCl instillation showed advanced protein-rich edema, capillary congestion, hemorrhage, and the presence of neutrophilic infiltrations and erythrocytes in alveolar spaces (Figure 1B). These findings ascertained the development of acute lung injury for the first hit.

3.2. Survival study

The survival rates of mice in the S + S, A + S, S + Ab, and A + Ab groups were 100%, 100%, 100%, and 0%, respectively, at 6 days (Figure 2). The survival rates of the S + S, A + S, and S + Ab groups were significantly higher than that of the A + Ab group (vs. A + Ab group, all $p < 0.05$ by log-rank test).

3.3. Bacterial loads in BALF

There was an increase in the bacterial loads in A + Ab mice, from 3.13×10^8 CFU/ml at 3 h to 1.63×10^{10} CFU/ml at 24 h, after Ab396 inoculation (Figure 3). In contrast, while a high initial bacterial titer of 4.73×10^7 CFU/ml was measured in the S + Ab mice at 3 h, there was a progressive reduction to 1.3×10^3 CFU/ml, nearly equal to that of the A + S and S + S mice, by 48 h. The bacterial titers of the A + Ab group were significantly higher than those of the S + S, A + S, and S + AB groups at 3 to 24 h (vs. S + S, A + S, and S + Ab mice, all $p < 0.05$ by Mann–Whitney *U*-test). The A + Ab mice suffered severe mortality, and very few mice survived to allow assessments beyond 48 h.

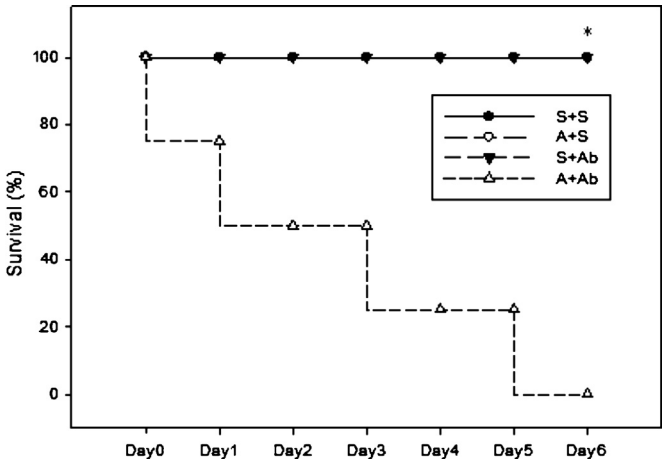

Figure 2. Survival rate of mice that were administered 0.1 N HCl intratracheally and a sub-lethal dose (1.0×10^8 CFU) of Ab396 (A + Ab), 0.9% saline and 1.0×10^8 CFU Ab396 (S + Ab), 0.1 N HCl and 0.9% saline (A + S), or 0.9% saline and 0.9% saline (S + S) ($n = 8$ for each group) during 6 days of follow-up.
*Significantly higher survival rates of the S + Ab, A + S, and S + S groups compared with the A + Ab group.

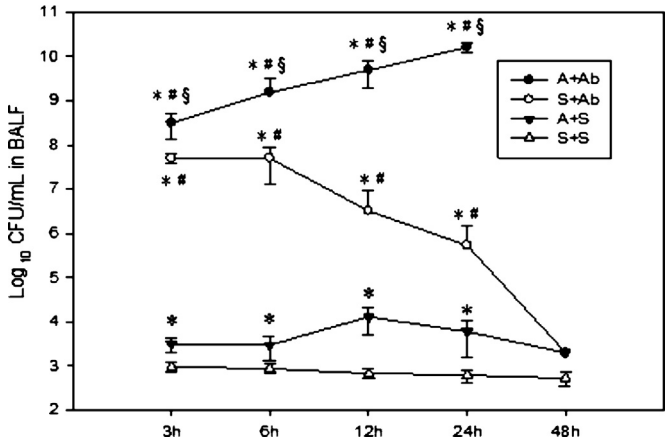

Figure 3. Time course analysis of bacterial loads of the BALF of mice that received 0.1 N HCl and 1.0×10^8 CFU of Ab396 (A + Ab), 0.9% saline and 0.9% saline (S+S), 0.9% saline and 1.0×10^8 CFU of Ab396 (S + Ab), or 0.1 HCl and 0.9% saline (A + S) ($n = 10$); data are expressed as the mean ± standard deviation.
*Significantly higher bacterial loads compared with the S + S group.
#Significantly higher bacterial loads compared with the A + S group.
§Significantly higher bacterial loads compared with the S + Ab group.

3.4. Cytokine profiles in BALF

The A + Ab mice had significantly lower levels of TNF-α than the S + Ab mice from 3 to 12 h (Figure 4A) (vs. S + Ab group, all $p < 0.05$ by Mann–Whitney *U*-test). In contrast, the IL-6, MIP-2, and IL-10 levels in the A + Ab mice were significantly higher than those in the S + S, A + S, and S + Ab groups from 3 to 24 h after infection (Figure 4, B–D) (vs. S + S, A + S, and S + Ab group, $p < 0.05$ for IL-6, IL-10, and MIP-2, by Mann–Whitney *U*-test).

3.5. Lung permeability and the interaction between acid and Ab396 inoculation

Extensive lung permeability was found in the A + Ab mice. There was a progressive increase in EBD titers in the A + Ab mice, from 3020.6 ± 934.1 μg/ml at 6 h to 10 875.9 ± 4519.2 μg/ml at 48 h, after Ab396 inoculation (Figure 5). The lung permeability of the A + Ab group was significantly higher than those of the S + S, A + S, and S + AB groups at 6, 24, and 48 h (vs. S + S, A + S, and A + Ab mice, $p < 0.05$ by Mann–Whitney *U*-test).

The interaction between acid induction and Ab396 inoculation for lung injury was also analyzed. At 6 h, a significant difference was found between the groups with and without acid treatment (vs. without acid group, $p < 0.05$ at 6 h by Mann–Whitney *U*-test, *p*-value of interaction < 0.05 at 6 h by two-way ANOVA), regardless of the presence or absence of bacterial infection. However, at 24 and 48 h, there was no significant difference in the lung permeability between the acid-treated and non-treated groups in the absence of Ab396, but there was a significant difference in the presence of Ab396 (vs. without Ab396 group, $p < 0.05$ at 24 and 48 h by the Mann–Whitney *U*-test, *p*-value of interaction < 0.05 at 24 and 48 h by two-way ANOVA). This result suggests a synergistic effect between acid environment and the presence of bacteria on the permeability from 6 to 48 h after treatment.

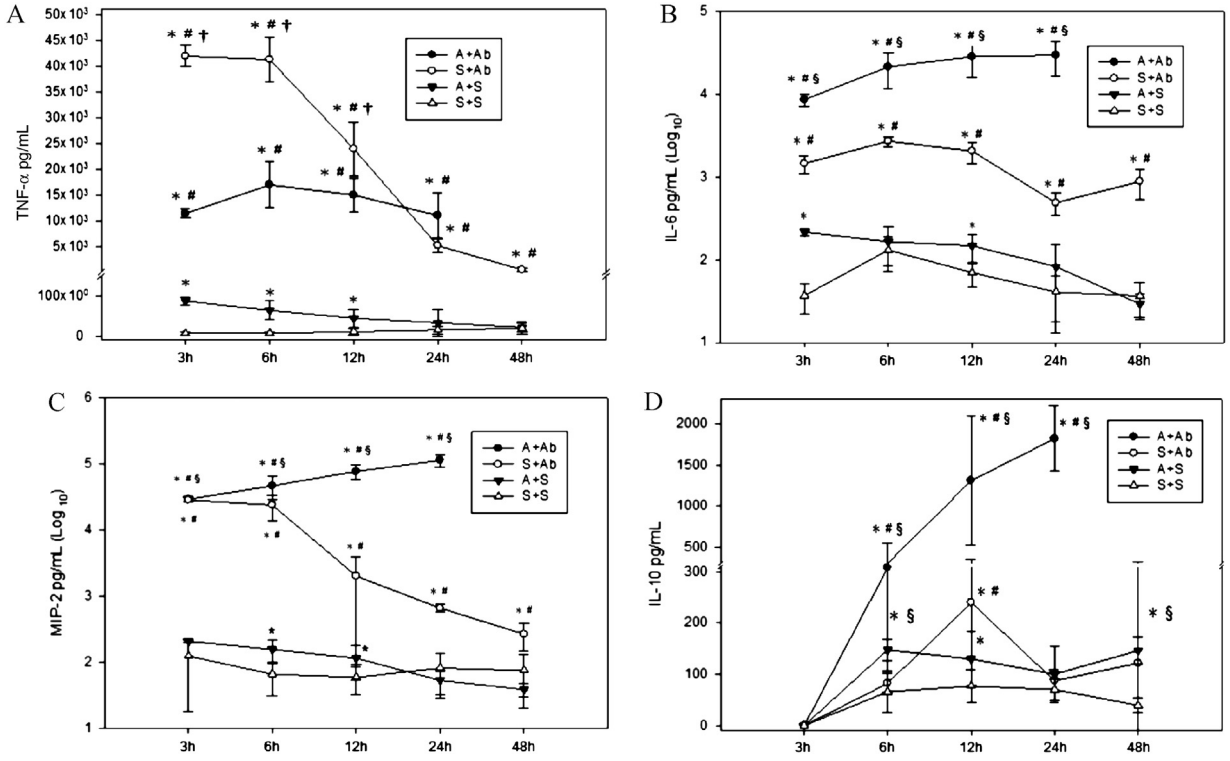

Figure 4. Cytokine profiles of (A) TNF-α, (B) IL-6, (C) MIP-2, and (D) IL-10 in BALF of mice that received 0.1 N HCl and 1.0×10^8 CFU of Ab396 (A + Ab), 0.9% saline and 1.0×10^8 CFU of Ab396 (S + Ab), 0.1 N HCl and 0.9% saline (A + S), or 0.9% saline and 0.9% saline (S + S). Figures illustrate time-course analyses of the TNF-α, IL-6, MIP-2, and IL-10 levels in the four groups ($n = 10$); data are expressed as the mean ± standard deviation.
†Significantly higher TNF-α levels compared with the A + Ab group.
*Significantly higher cytokine levels compared with the S + S group.
#Significantly higher cytokine levels compared with the A + S group.
§Significantly higher cytokine levels compared with the S + Ab group.

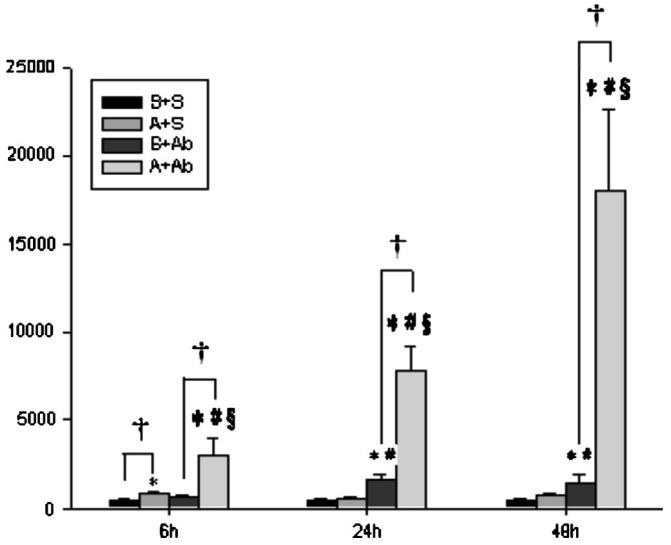

Figure 5. Time-course analysis of the lung permeability and the interaction between the acidic environment and Ab396 infection in the mice that received 0.1 N HCl and 1.0×10^8 CFU of Ab396 (A + Ab), 0.9% saline and 1.0×10^8 CFU of Ab396 (S + Ab), 0.1 N HCl and 0.9% saline (A + S), or 0.9% saline and 0.9% saline (S + S) ($n = 8$ per group); data are expressed as the mean ± standard deviation.
*Significantly higher Evans blue levels compared with the S + S group.
#Significantly higher Evans blue levels compared with the A + S group.
§Significantly higher Evans blue levels compared with the S + Ab group.
†Significantly higher Evans blue levels of the 0.1 N HCl or Ab396-treated groups compared with the groups not given acid or Ab396 for the interaction study.

3.6. Histopathological studies of the lungs

The histopathology of the lungs of the tested mice is shown in Figure 5. Mild infiltrations of polymorphonuclear leukocytes (PMNs) into the pulmonary interstitium were observed in the A + S (Figure 6A) and the S + Ab groups (Figure 6B). In contrast, the infected lungs of the A + Ab group displayed advanced consolidation, acute alveolar inflammation, and extensive infiltration of PMNs in the interstitium (Figure 6C). The intact alveolus with no PMN infiltration from a healthy mouse is shown in Figure 6D.

4. Discussion

Our results demonstrate that 0.1 N acid aspiration directly induces lung injury, and the ensuing interaction with Ab396 leads to secondary pneumonia in mice. To our knowledge, this is the first study to investigate the causal association between acid aspiration and the development of MDRAB pneumonia in vivo. These novel findings may provide important insights into the pathogenesis and prevention of MDRAB pneumonia.

The pathogenesis of secondary pneumonia after acid aspiration has been investigated in previous experimental studies. Mitsushima et al. infected mice with *Pseudomonas aeruginosa* 5 min after IT delivery of 0.1 N HCl,[21] but did not assess the inflammatory responses of the lungs. Rotta et al. infected rats with *Escherichia coli* 1 min after IT gastric aspiration and reported transient increases in TNF-α, IL-1β, and MIP-2, but a more sustained elevation of IL-10.[24] Additionally, van Westerloo and colleagues infected mice with *Klebsiella pneumoniae* 16 h after IT administration of 0.1 N HCl and observed increased levels of TNF-α, IL-10, IL-6, MIP-2, and IL-1β.[25] Our study differs from these earlier investigations, not only because we used an opportunistic pathogen, but also because we chose the 5-min time point for the bacterial challenge. Of note, we also made an important novel observation of early down-regulation of TNF-α levels in the BALF of mice with Ab396 pneumonia when compared with the S + Ab mice. These findings demonstrate that Ab396 pneumonia following acid aspiration has important immunological impacts on the host's defense that differ from the secondary pneumonia caused by other pathogens,[25] in addition to primary *A. baumannii* pneumonia in which increased TNF-α expression has been reported.[26]

Our results demonstrate differences in the immunological responses between the S + S, A + S, and S + Ab groups. Specifically, the IL-10, MIP-2, and IL-6 levels were significantly higher in the A + Ab mice compared with the mice in the other three groups, whereas the TNF-α levels were significantly lower than those in

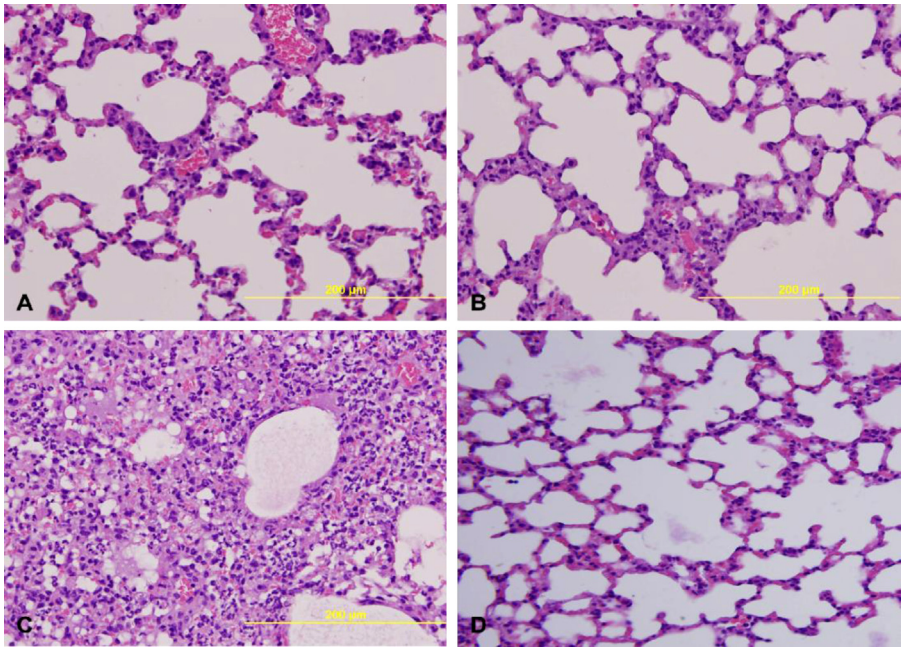

Figure 6. Histopathologic changes of lungs in the mice that received (A) 0.1 N HCl and 0.9% saline (A + S), (B) 0.9% saline and 1.0×10^8 CFU of Ab396 (S + Ab), (C) 0.1 N HCl and 1.0×10^8 CFU of Ab396 (A + Ab), sacrificed at 48 h post-infection, as well as (D) healthy mice; hematoxylin and eosin stain.

the S + Ab group at early time points (Figure 4). This may indicate a shift towards a weakened inflammatory response with lower TNF-α levels and higher IL-10 levels in A + Ab mice compared with the S + Ab mice.

TNF-α has a significant role in the host's innate pulmonary defense against bacterial pathogens.[27,28] Low levels of TNF-α in the lungs may suggest impairment of the host's antibacterial responses. Moreover, excessive expression of IL-10 could lead to less efficient bacterial clearance, in part by a down-regulation of TNF-α.[29–31] Additionally, high IL-10 levels in BALF were strongly correlated with lung injury and the inhibition of bacterial clearance in pneumonia following acid aspiration.[24,32] Thus, low TNF-α and high IL-10 levels may contribute to the detrimental outcome observed in the A + Ab mice, as demonstrated by a strong correlation between high levels of anti-inflammatory mediators and poor outcomes.[33,34] In contrast, up-regulation of TNF-α has been reported in animals following acid aspiration and secondary pneumonia caused by other pathogens.[25,35] However, the TNF-α level may not necessarily equate to the severity of the inflammatory reaction because anti-TNF-α therapy exhibits poor efficacy in the treatment of secondary pneumonia.[25] Also, the finding of up-regulation of MIP-2 levels is consistent with previous reports,[36] and this may play an important role in lung injury after acid aspiration. Given the complexity of the immune mechanisms responsible for protection against pathogens causing secondary pneumonia, this immunopathogenesis requires further investigation.

The precise pathogenesis of the deficient TNF-α and high IL-10 responses in the A + Ab mice has not been fully explored. One possibility is the up-regulation of receptor or signaling of toll-like receptor 2 (TLR2) in vivo to decrease the TNF-α expression, as Knapp et al. reported higher up-regulated TNF-α responses in $TLR2^{-/-}$ mice after infection with *A. baumannii* compared with those in $TLR4^{-/-}$ mice.[26] Another possibility is that up-regulation of IL-10 expression results in the attenuation of proinflammatory cytokine levels to prevent systemic over-inflammation for the immune system.[29–31] It occurs in a condition of compensatory anti-inflammatory response syndrome (CARS);[37,38] in fact numerous studies of 'two-hit' animal models have found a positive correlation between the severity of CARS and poor outcomes in secondary bacterial infections.[37]

It is important to note that without the initial lung permeability induced by the preceding acid treatment, Ab396 inoculation alone could not induce the extensive damaging effects observed in the pneumonia. Thus, the initiation of lung injury by 0.1 N acid aspiration is indispensable to provoke the secondary Ab396 pneumonia (Figure 5). Furthermore, neither the acid treatment nor the Ab396 inoculation alone could establish the fatal effect observed in the tested mice. Very little is known about the interactions between *A. baumannii* infection and acid insult in lung injury. Thus the interaction of the acidic environment and Ab396 infection was evaluated. These results illustrate that the joint effect of the acidic environment and the presence of Ab396 exceeded the sum of their individual effects in the causation of lung injury in A + Ab mice (Figure 5). Therefore, a combination of acid plus Ab396 caused lung injury that was synergistically more severe than that from either 0.1 N acid or Ab396 alone. This finding differs from those of a previous report, in which only an acidic environment contributed to lung injury due to *E. coli* pneumonia following gastric aspiration.[24] The link between preexisting acid aspiration and the subsequent occurrence of MDRAB pneumonia merits further evaluation.

In conclusion, our results highlight three major findings: (1) lung injury induced by 0.1 N acid aspiration facilitates the development of secondary Ab396 pneumonia; (2) weak TNF-α expression in BALF may contribute to the decreased survival in A + Ab mice; and (3) the synergistic interaction between the acidic environment and Ab396 infection enhanced the lung injury. These data strongly support the hypothesis that acid aspiration is one of multiple factors that can lead to secondary MDRAB pneumonia in mice.

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Ethical approval: This study was approved by the Institutional Animal Care and Use Committee (IACUC); IACUC Approval No. 94122202.

Conflict of interest: The authors report that no potential conflicts of interest exist with any companies or organizations.

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